treatment was extended to 40 min (Figure 1D). In hatched eggs, 20-min treatment with NaOCl exposed a layer of fine, vertically oriented crystals, patches of which broke free and revealed a deeper, more stable surface (Figure 1G); 40-min treatment removed all fine crystals, and the deeper layer appeared to have a negative image of the balanoid facets (Figure 1H).

DISCUSSION

Calcium dynamics in somatic and reproductive physiology have been studied in snails nominally assigned to *Pomacea paludosa* (Meenakshi *et al.,* 1974, 1975; Watabe *et al.,* 1976; Meenakshi & Watabe, 1977). The snails differ, however, from *P. paludosa* in egg size, capsular ultrastructure, clutch size and morphology, hatching time, and protoconch size (Perry, 1973; McCabe & Turner, unpublished data). Moreover, they differ in calcium dynamics during embryogenesis (see below). In the absence of voucher specimens (Watabe, pers. comm.), we only speculate that their material from local pet shops (Meenakshi *et al.,* 1974) was the Brazilian *P. bridgesi* (Reeve, 1856), which is marketed commercially (Thompson, 1984; Starmühlner, 1989).

Members of the Pilidae, Neritidae, and 36 families of pulmonates lay terrestrial eggs with calcified capsules (Tompa, 1980). In reviewing available information on embryonic calcium dynamics in gastropods, Tompa (1980) concluded that calcium for the hatchling is derived predominately from the capsule in cleidoic, calcified eggs and from extracapsular sources in non-cleidoic, poorly or uncalcified eggs. In *Anguispira alternata* (Say, 1816), the intracapsular contents at oviposition contain only 1% of the calcium of the 43-day embryo, and calcium content of the capsule is correspondingly reduced during embryogenesis (Tompa, 1975). Only 6.7% of the calcium of the protoconch is attributable to the intracapsular fluids of *Pomacea* sp. (Meenakshi & Watabe, 1977). Hatchling *Veronicella ameghini* (Gambetta, 1923) derives 22% of its calcium from intracapsular fluids (Tompa, 1980). In the present study, on the other hand, we found that embryonic calcium requirements of *P. paludosa* are met entirely from intracapsular sources. Without clear presentation of data or methodology, Fournié & Chétail (1984) indicated that *Deroceras reticulatum* (O. F. Müller, 1774) also is independent of capsular calcium; but Bayne's (1966) equally brief account of a more qualitative analysis reported a reduction of capsular calcium.

Tompa (1980) hypothesized that an osmotic or other toxic effect precluded the storage of sufficient intracapsular calcium to support embryogenesis of cleidoic eggs. But intracapsular calcium concentrations can be high in newly oviposited eggs: 3.8 m*M* in *Veronicella ameghini* (Tompa, 1980); 9.1 m*M* in *Anguispira alternata* (calculated from Tompa [1975]); 37 m*M* in *Deroceras reticulatum* (Fournié & Chétail, 1984); 183 m*M* in *Pomacea* sp. (calculated from Meenakshi *et al.* [1974: fig. 8] and Meenakshi & Watabe [1977]); 745 m*M* in *Pomacea paludosa* (present study). Alternatively, Fournié & Chétail (1984) predicted that most of the calcium in intracapsular fluids is organically bound; this would seem to be the case for all species cited above if physiologic concentrations of free calcium are typically 10^{-3} m*M* (Eckert *et al.,* 1988). The calcium-rich egg of *P. paludosa* represents an extreme in the range of strategies by which gastropods provide calcium for embryogenesis.

In addition to chemical analyses of capsules and intracapsular material, evidence for calcium resorption from the capsule comes from direct observation of changes in its crystalline structure. The number of calcium carbonate spherules in the partly calcified capsule of *Veronicella ameghini* decreases during embryogenesis (Tompa, 1980). Tompa (1979) demonstrated an observable loss of crystals in the capsule of *Stenotrema leaii* (Ward *in* Binney, 1840) and reported similar losses to occur in *Varohadra yeppoonensis* (Beddome, 1897) and *Helicodiscus parallelus* (Say, 1821). Heavy erosion of the initially thick inner crystalline layer in *Pomacea* sp. progresses to the thin, outer monolayer of spherules by the time of hatching (Meenakshi & Watabe, 1977). Erosion in *P. paludosa,* on the other hand, is superficial and does not contribute measurably to calcium dynamics of the embryo; changes in surface morphology and structural integrity of the inner capsular layer might only serve to weaken the capsule in preparation for hatching.

Reliance on eggshell calcium for embryogenesis has been demonstrated in insects (Moscona, 1948), gastropods (Tompa, 1980), reptiles (Packard *et al.,* 1977), and birds (Bond *et al.,* 1988). The degree of reliance, however, varies widely. For example, embryos of altricial birds erode the eggshell mammillae less than do embryos of precocial birds (Bond *et al.,* 1988); and squamate reptiles rely on calcium stores in the yolk, whereas crocodilians and chelonians rely on eggshell calcium (Packard *et al.,* 1977). Mechanisms for calcium provision in terrestrial eggs of gastropods are more diverse (Tompa, 1980) than for avian and reptilian eggs. The present study extends this diversity among gastropods further to include total reliance on calcium stores in the intracapsular fluids, a mechanism previously considered maladaptive (Tompa, 1980).

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Ca-Binding Glycoproteins in Molluscan Shells with Different Types of Ultrastructure

by

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Abstract. Highly acidic Ca-binding glycoproteins were isolated from molluscan shells that have various types of ultrastructure. The molecular weights of the proteins were estimated to range between approximately 50,000 and 70,000 daltons and all were rich in acidic amino acids (particularly aspartate) and in glycine and serine. Based on the results of the amino acid analyses, Ca-binding glycoproteins could be classified into three major types: (1) those of the nacreous and prismatic layers; (2) those of the foliated and chalky layers; and (3) those of the homogeneous, composite-prismatic, complex, and crossed-lamellar layers.

INTRODUCTION

Molluscan shells consist primarily of crystals of calcium carbonate ($CaCO_3$), generally either aragonite or calcite. Depending on the arrangement of crystals, calcified layers of shells are classified into different types such as the granular, homogeneous, nacreous, prismatic, foliated, composite-prismatic, complex, and crossed-lamellar layers (MacClintock, 1967; Taylor *et al.*, 1969; Kobayashi, 1971; Carter, 1980; Carter & Clark, 1985; Uozumi & Suzuki, 1981). Small amounts of organic materials are also present in shells, as a periostracum, covering the outer surfaces of shells, and as an organic matrix in mineralized layers.

The organic matrix, which is a mixture of protein, glycoprotein, polysaccharide and lipid (Wada, 1964, 1980; Degens, 1979; Krampitz & Witt, 1979; Samata & Krampitz, 1981), is composed of two components: a water-soluble matrix (SM) and a water-insoluble matrix (ISM). A soluble matrix was first noted by Crenshaw (1972) in shells of *Mercenaria mercenaria*. Because of the Ca-binding capacity of the SM, it is considered essential for the process of shell formation. An insoluble matrix, which corresponds to "conchiolin" of Frémy (1855), constitutes the main part of the matrix and is thought to be the framework of the matrix. According to Grégoire (1972), the amino acid composition of the ISM differs among various taxa of mollusks. On the other hand, Akiyama (1966) and Kasai & Ohta (1981) found a more-or-less unique amino acid composition of the ISM for each type of shell ultrastructure, regardless of taxon. Moreover, Weiner (1983) fractionated the SM of the nacreous and prismatic layers of *Mytilus californianus* by HPLC and reported that the amino acid sequence of Asp-Pro-Thr-Asp is uniquely found in the calcitic prismatic layer. Although molecules in the organic matrix are considered to be laid down with a particular organization prior to the formation of crystals of $CaCO_3$, the origin and function of the organic matrix have not been clarified.

In the present study, amino acid compositions of the SM in various types of molluscan shell ultrastructures were determined, in order to correlate the composition, shell ultrastructure, and taxonomy of mollusks. For this purpose, the matrices were isolated from eight types of shell layers representing 11 molluscan families and three classes. After the Ca-binding components were fractionated from the bulk SM by two steps of column chromatography, the amino acid compositions of the Ca-binding components were compared.

MATERIALS AND METHODS

The taxonomies, localities of origin, and shell ultrastructures of the species examined are shown in Table 1.

Figure 1 shows a flow diagram of the preparation and analysis of the SM. After their surfaces were cleaned with a dental drill, shells were dipped in 1% NaClO to remove organic contaminants and periostracum. Individual layers of a shell were separated with a dental drill, dried, powdered, and decalcified. Decalcification was carried out in 1 *N* HCl and the salts were removed by dialysis against distilled water in Spectrapor No. 3 tubing (Spectrum Med-

Table 1

Taxonomic allocations, shell ultrastructures, and localities of origin of the 12 species examined. N, nacreous layer; P, prismatic layer; F, foliated layer; CH, chalky layer; H, homogeneous layer; CP, composite-prismatic layer; C, complex layer; CL, crossed-lamellar layer.

Taxonomy	Shell ultra-structure	Locality of origin
A. Gastropoda		
Haliotidae		
Haliotis discus Reeve	N, P	Sesoko Jima, Okinawa
Turbinidae		
Turbo cornutus Solander	N, P	Miura Shi, Kanagawa
B. Bivalvia		
Pteriidae		
Pinctada martensii Dünker	N, P	Omura Shi, Nagasaki
Pinnidae		
Atrina vexillum Born	N, P	Omura Shi, Nagasaki
Placunidae		
Placuna placenta Linné	F	Philippine
Pectinidae		
Patinopecten yessoensis Jay	F	Mutsu Shi, Aomori
Ostreidae		
Crassostrea gigas Thunberg	F, CH	Matsushima, Miyagi
Arcidae		
Anadara broughtonii Schrenck	C, CL	Misaki Shi, Kanagawa
Glycymeridae		
Glycymeris yessoensis Sowerby	C, CL	Mutsu Shi, Aomori
Veneridae		
Mercenaria stimpsoni Gould	H, CP	Georgetown, SC, USA
Meretrix lusoria Röding	H, CP	Kujukuri, Chiba
C. Cephalopoda		
Nautilidae		
Nautilus pompilius Linné	N, P	Kuro Shima, Okinawa

ical Industries Inc., New York, U.S.A.). Only molecules of molecular weight less than 3500 daltons passed through the pores of this dialysis tube. Each dialyzed sample was centrifuged (10,000 × g, 20 min). Only the supernatant was used for further fractionation.

An aliquot of 0.1 mL radioactive $^{45}CaCl_2$ (0.05 μCi) was added to each 2 mL of water-soluble sample before fractionation on a column of Bio-Gel A 1.5 m. Radioactivity was measured in a Packard Tricarb Model 4530 liquid scintillation counter (Packard Instrument Co., New York, U.S.A.).

The SM was initially loaded onto a 100 × 1.5 cm, Bio-Gel A 1.5 m column (Bio-Rad Laboratories, Tokyo, Japan), equilibrated with 0.1 *M* NH_4HCO_3, pH 8.4. The absorbance of the eluate was measured at 235 nm with a double beam spectrophotometer UV 150-02 (Shimazu Co., Kyoto, Japan). The Ca-binding fractions in the eluate were pooled, lyophilized, and subjected to ion exchange chromatography on a column (30 × 2.5 cm) of DEAE Sephacel (Pharmacia Japan Co., Tokyo, Japan), eluted with 0.1 *M* NH_4HCO_3 buffer and then with a linear gradient from 0 to 1 *M* NaCl in the same buffer. All fractions were pooled and desalted on a Bio-Gel P 6 column (50 × 0.9 cm), eluted with 0.01 *M* NH_4HCO_3. For precise determination of the molecular weight, material was run on columns of DEAE Sephacryl S-200 and Sephadex G 75 (Pharmacia Japan Co.; 100 × 1.5 cm), respectively, in the same buffer as used for chromatography on Bio-Gel A 1.5 m.

The purified Ca-binding fractions were hydrolyzed in a hydrolysis tube (Pierce Co., Chicago, U.S.A.) under vacuum, at 110°C for 24 h, in 6*N* HCl. The hydrolysates were then analyzed on an Atto MLC-703S automatic amino acid analyser (Atto Co., Tokyo, Japan).

Analysis was repeated for three Ca-binding fractions from different specimens of the same species.

SDS-Polyacrylamide gel electrophoresis was carried out in 12.5% gels as described by ANDERSON *et al.* (1983) and isoelectric focusing in ampholine (pH 3.0 to 10.0) as described by O'FARRELL (1975). Both gels were stained with 0.4% Coomassie Brilliant Blue R-250. Samples for electrophoresis were prepared in three concentrations (10, 50, and 100 μg/50 μL sample buffer).

Infrared spectroscopic analysis was performed by the diffuse reflectance method on an IR-810 DP 98 spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan).

Quantitative analysis of phosphate was carried out by the method of TAUSSKY & SCHORR (1967) and of hexose by the method of SCHIELDS & BURNET (1960).

RESULTS

Total Amount of SM

The amount of SM was low, ranging from 0.01 to 0.2% of the total weight of each shell. The amount of the SM in the prismatic and foliated layers was the highest; the next highest level was found in the nacreous layer. The other four layers of shell had even lower amounts of SM.

Gel Chromatography

Elution profiles of SM obtained by Bio-Gel A 1.5 m are shown in Figure 2 (left). Sample species are *Pinctada martensii* for the nacreous and prismatic layers, *Crassostrea gigas* for the foliated layer, and *Glycymeris yessoensis* for the crossed-lamellar layer. Regardless of the type of ultrastructure, the largest amount of protein was eluted from the column with 60 to 90 mL of the buffer. This major fraction included about 70 to 80% of the total protein in

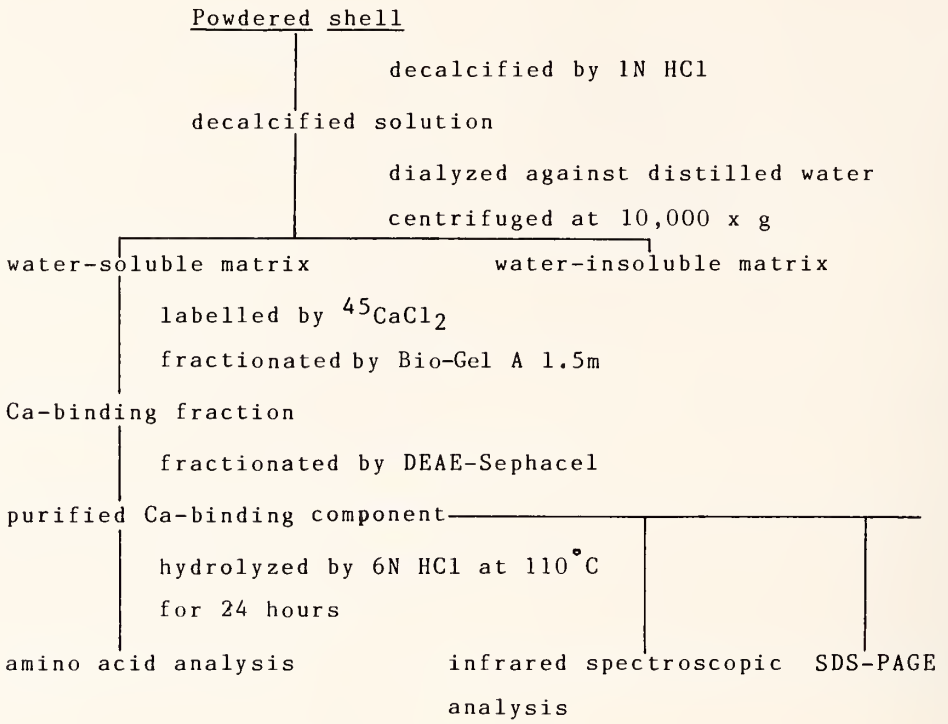

Figure 1

Flow-diagram of the procedure used for analysis of the water-soluble matrix.

the matrix and the eluted material had Ca-binding capacity. Rechromatography of the major protein fractions on DEAE Sephacryl S-200 and Sephadex G 75 showed essentially the same elution profiles for all species with major molecular weights ranging from about 50,000 to 70,000 daltons.

Ion Exchange Chromatography

Elution profiles after ion exchange chromatography (DEAE Sephacel) of the main components isolated by gel chromatography are shown in Figure 2 (right). The largest amount of protein was eluted by 0.5 to 0.6 *M* NaCl in the case of the prismatic, nacreous, and foliated layers, and at about 0.4 *M* NaCl in the case of the other four layers.

Ca-binding capacity could be detected only in the major acidic fractions of all species examined.

Amino Acid Composition

Amino acid compositions of the Ca-binding components in the nacreous and prismatic layers (Table 2), and in the foliated, chalky, homogeneous, composite-prismatic, complex, and crossed-lamellar layers (Table 3), are shown. The value for each amino acid corresponds to the mean value obtained from the analyses of three specimens for each species. Although some distinctive differences can be recognized in the amino acid compositions of shells of different ultrastructures, the compositions were generally characterized by high levels of aspartate, glycine, glutamate, serine, and alanine. Acidic amino acids were found to be present at higher levels than basic amino acids. Polar amino acids were also present in high amounts.

Table 4 shows the mean value and the standard deviation of levels of each amino acid in the Ca-binding components isolated from the six shell layers examined. The mean value and the standard deviation were calculated for a total of five species in the case of both the nacreous and prismatic layers, three of the foliated layer, and two of the remaining three layers.

Compositions of the Ca-Binding Components in the Nacreous and Prismatic Layers

Although the nacreous layer is composed of aragonite, the prismatic layer is of calcite (in Gastropoda and Bivalvia) or aragonite (in Cephalopoda). As clearly indicated in Table 4, the range of variation of each amino acid (mean value + standard deviation) overlapped between the nacreous and prismatic layers for almost all amino acids. The values were somewhat different only in the case of alanine. This result shows the high degree of similarity in the amino acid composition of the Ca-binding components in the two ultrastructural layers. The composition was characterized by large amounts of aspartate and glycine, which com-

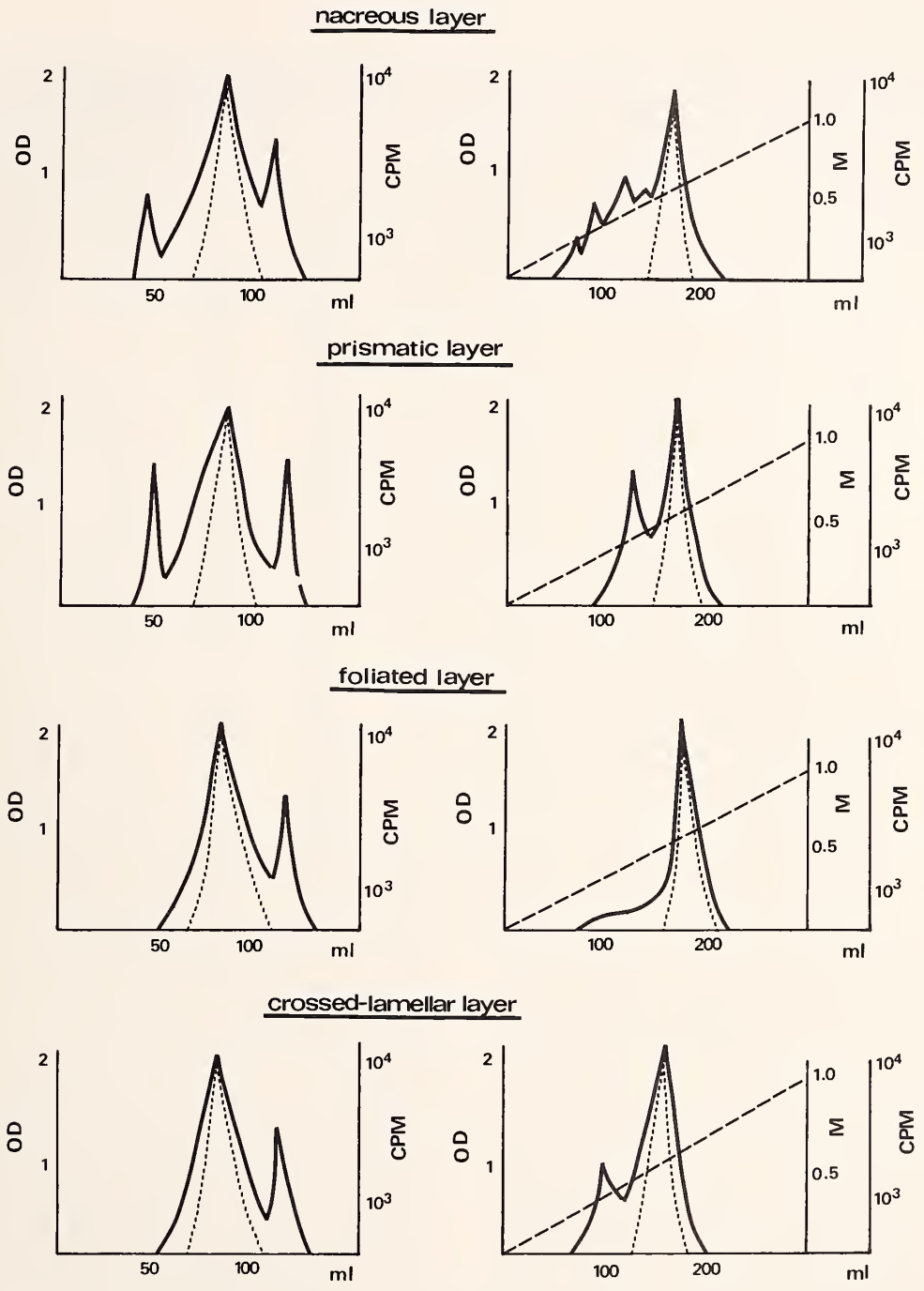

Figure 2

Elution profiles of the water-soluble matrix in each shell layer obtained by Bio-Gel A 1.5 m (left) and DEAE Sephacel (right). Species: nacreous and prismatic layers, *Pinctada martensii*; foliated layer, *Crassostrea gigas*; crossed-lamellar layer, *Glycymeris yessoensis*. Key: -----, capacity of Ca-binding; – – –, concentration of NaCl; ——, absorbance at 235 nm. Absorbance at 235 nm (OD) is marked by the scale on the left margin of each profile; intensity of Ca-binding (CPM) and the salt gradient (*M*) is marked on the right margin of each profile.

Table 2

Amino acid compositions of the Ca-binding glycoproteins in the nacreous and prismatic layers (in molar percent). 1, *Haliotis discus*; 2, *Turbo cornutus*; 3, *Pictada martensii*; 4, *Atrina vexillum*; 5, *Nautilus pompilius*; 1 and 2 are gastropods, 3 and 4 are bivalves, and 5 is a cephalopod. —, not detected; a.a., amino acids.

Layer:	Nacreous					Prismatic				
Species:	1	2	3	4	5	1	2	3	4	5
Asx	26.55	27.46	26.38	26.79	29.23	29.53	26.40	28.95	28.55	25.58
Thr	3.44	5.70	3.12	5.13	3.31	2.95	3.72	3.03	4.81	3.33
Ser	6.22	7.30	6.48	8.15	4.98	8.00	9.15	10.75	9.09	3.96
Glx	8.87	9.72	10.73	9.56	9.44	8.41	11.27	9.42	10.59	9.55
Pro	6.81	6.62	6.24	4.24	6.17	4.82	5.40	5.10	5.14	5.01
Gly	22.48	15.41	23.15	19.55	17.75	25.46	17.63	21.54	17.99	18.68
Ala	6.47	7.58	6.58	5.95	7.40	4.10	3.96	5.28	5.81	2.56
Cys	0.81	0.21	1.06	1.46	0.50	0.50	1.98	1.29	0.63	—
Val	3.59	3.54	2.52	2.93	4.41	2.07	2.62	2.80	2.78	2.77
Met	1.46	1.26	0.20	0.85	0.33	—	—	0.13	—	0.42
Ile	1.66	2.00	1.27	1.68	2.66	0.81	1.62	1.56	1.91	1.99
Leu	3.29	3.95	2.84	2.84	4.89	2.29	2.98	3.55	3.34	2.60
Tyr	1.01	1.55	2.35	1.57	1.16	2.16	1.65	0.80	1.72	9.18
Phe	1.98	2.52	3.01	2.77	2.55	2.44	3.83	1.46	2.24	3.00
Lys	3.76	2.19	1.80	2.62	1.81	3.14	2.62	1.33	2.07	5.49
His	0.40	1.00	0.43	1.21	1.02	0.57	1.84	0.96	1.47	2.21
Arg	2.82	1.78	1.84	2.70	2.39	2.75	3.33	2.05	1.86	3.67
Acidic a.a.	35.42	37.18	37.11	36.35	38.67	37.98	37.67	38.37	39.14	35.13
Basic a.a.	6.98	4.97	4.07	6.53	5.22	6.46	7.79	4.34	5.40	11.37
Acidic/basic	5.07	7.48	9.12	5.57	7.41	5.88	4.84	8.84	7.25	3.09
Asx/Glx	2.99	2.83	2.46	2.80	3.10	3.51	2.34	3.07	2.70	2.68
Hydroxy a.a.	9.66	13.00	9.60	13.28	8.29	10.95	12.87	13.78	13.90	7.29
Ser/Thr	1.81	1.28	2.08	1.59	1.50	2.71	2.46	3.55	1.89	1.19
Gly/Ala	3.47	2.03	3.52	3.29	2.40	6.21	4.45	4.08	3.10	7.30
Asx/Gly	1.18	1.78	1.14	1.37	1.65	1.26	1.50	1.34	1.59	1.37
Polar a.a.	75.36	72.32	77.34	78.74	71.59	83.51	79.61	80.12	78.78	81.65

prised from 26 to 30 mole percent (%) for aspartate and 15 to 25% for glycine. The level of glutamate was also high, being about 10% or 40% higher than in the foliated layer. Among the hydroxy amino acids, serine was lower in concentration and threonine higher in the nacreous and prismatic layers than in the foliated layer. Acidic amino acids were present at higher levels than basic amino acids in both layers. Proline, which was present in only small amounts in the foliated layer, was found at concentrations about 2- to 3-fold greater in the nacreous and prismatic layers. Apart from these amino acids, the amounts of valine, isoleucine, leucine, phenylalanine, lysine, and histidine were slightly higher than in the foliated layer. Among aromatic amino acids, phenylalanine was usually present in higher levels than tyrosine, but this trend was reversed in the foliated layer.

For a more precise comparison between the nacreous and prismatic layers, the ratio of each amino acid residue in the Ca-binding components in the nacreous layer to that in the prismatic layer was calculated for five species. The results show a distinct increase in the amount of alanine and a decrease in the amount of histidine and of polar amino acids in the nacreous layer as compared to the prismatic layer. Moreover, serine residues were more concentrated in the prismatic layer. These trends were found in almost all the species examined.

In contrast to the similar compositions of the Ca-binding components of the specimens of Gastropoda and Bivalvia, the composition of the cephalopod *Nautilus pompilius* was unique with respect to several amino acids. In particular, the level of serine was low, about 25% lower in the nacreous layer and 50% lower in the prismatic layer than in the other species. Moreover, the extremely low level of alanine, the high levels of tyrosine and lysine, and the low ratio of serine to threonine were also characteristic of the prismatic layer of this species. The unique composition of *N. Pompilius* is in large measure responsible for the high value of the standard deviations of serine, tyrosine, and lysine for the prismatic layer of the five species examined (see Table 4).

Compositions of the Ca-Binding Components in the Foliated and Chalky Layers

The amino acid compositions of the Ca-binding components in the calcitic foliated and chalky layers were similar, characterized by high levels of aspartate, serine,

Table 3

Amino acid compositions of the Ca-binding glycoproteins in the foliated, chalky, homogeneous, composite-prismatic, complex, and crossed-lamellar layers (in molar percent). 6, *Placuna placenta*; 7, *Patinopecten yessoensis*; 8, *Crassostrea gigas*; 9, *Mercenaria stimpsoni*; 10, *Meretrix lusoria*; 11, *Anadara broughtonii*; 12, *Glycymeris yessoensis*; all are bivalves. —, not detected; a.a., amino acids.

Layer:	Foliated			Chalky	Homogeneous		Composite-prismatic		Complex		Crossed-lamellar	
Species:	6	7	8	8	9	10	9	10	11	12	11	12
Asx	19.50	26.92	32.65	31.85	12.55	14.67	16.50	18.93	11.86	12.21	10.86	12.24
Thr	1.38	2.11	2.26	2.16	7.88	7.20	7.89	6.83	4.62	5.75	5.06	5.30
Ser	20.37	18.12	16.75	19.44	8.82	6.95	7.79	8.00	5.40	5.90	5.33	5.36
Glx	4.86	6.16	5.97	6.02	11.25	10.13	12.46	10.50	9.49	10.66	11.28	11.66
Pro	1.34	2.86	1.24	1.37	8.71	9.33	10.39	8.87	17.34	16.61	15.85	13.82
Gly	32.80	26.24	27.37	26.33	9.45	9.85	13.36	13.50	16.09	13.63	15.67	12.68
Ala	5.54	7.43	4.59	3.84	6.70	5.57	8.47	7.24	12.68	11.47	12.02	10.35
Cys	0.14	0.42	1.26	1.06	1.51	2.02	1.14	0.88	0.22	1.25	0.60	0.96
Val	1.61	0.97	0.88	0.87	5.74	4.62	3.90	4.54	4.90	4.46	5.03	5.35
Met	—	—	—	—	0.57	1.09	0.25	—	0.44	0.11	0.62	0.74
Ile	0.48	0.25	0.23	0.55	3.44	3.64	1.88	3.00	2.93	2.73	2.94	3.80
Leu	1.80	2.82	1.50	1.18	4.05	3.83	4.12	4.31	4.52	3.97	4.97	5.11
Tyr	3.05	1.44	2.59	2.61	3.03	3.42	2.58	2.00	0.95	0.91	0.07	1.03
Phe	0.84	0.51	0.18	0.90	2.61	4.00	2.04	2.50	2.31	3.00	2.56	3.01
Lys	0.84	1.63	1.10	0.85	7.53	8.38	4.75	5.10	2.88	4.27	3.16	4.43
His	0.41	0.31	0.25	0.09	3.84	2.40	1.10	1.30	0.87	0.85	0.53	0.67
Arg	3.54	1.81	1.18	0.88	2.32	2.88	1.38	2.56	2.52	2.22	3.44	3.49
Acidic a.a.	24.36	33.08	38.62	37.89	23.80	24.80	28.96	29.43	21.35	22.87	22.14	23.90
Basic a.a.	4.97	3.75	2.53	1.82	13.69	13.66	7.23	8.96	6.27	7.34	7.13	8.59
Acidic/basic	5.09	8.82	15.26	20.82	1.74	1.82	4.01	3.28	3.41	3.12	3.11	2.78
Asx/Glx	4.01	4.37	5.47	5.29	1.12	1.45	1.32	1.80	1.25	1.15	0.96	1.05
Hydroxy a.a.	21.75	20.23	19.01	21.60	16.70	14.15	15.68	14.83	10.02	11.65	10.39	10.66
Ser/Thr	14.76	8.59	7.41	9.00	1.12	0.97	0.99	1.17	1.17	1.03	1.05	1.01
Gly/Ala	5.92	3.53	5.96	6.86	1.41	1.77	1.58	1.86	1.27	1.19	1.30	1.23
Asx/Gly	0.59	1.03	1.19	1.21	1.33	1.49	1.24	1.40	0.74	0.90	0.69	0.97
Polar a.a.	86.89	85.16	91.38	91.31	68.18	67.90	68.95	70.18	54.90	57.65	56.00	57.82

and glycine, which constituted 70 to 80% of the total amino acid residues. In particular, the level of serine was 2- to 4-fold greater than in the remaining shell layers. As a result of such high proportions of these three amino acids, polar amino acids together represented about 85 to 91% of total residues. Moreover, the ratios of acidic to basic amino acids and of serine to threonine were also characteristically high. Of the other amino acids eluting after alanine from the column, cystein, isoleucine, phenylalanine, lysine, and histidine were found, but only in very small amounts.

The amounts of aspartate, serine, and glycine varied in the three species examined. Aspartate was found at its highest level in *Crassostrea gigas* and at its lowest in *Placuna placenta*, while the highest levels of serine and glycine were found in the latter species.

The standard deviation of levels of aspartate among the three species was 2-fold greater than the value among the three specimens of each species. In addition, the standard deviation in the levels of aspartate among the three species was also slightly greater than those in the levels of serine and glycine.

Compositions of the Ca-Binding Components in the Homogeneous, Composite-Prismatic, Complex, and Crossed-Lamellar Layers

The amino acid compositions of the Ca-binding components in these four shell layers of aragonite were different from those of the nacreous, prismatic, foliated, and chalky layers, in terms of the greater levels of proline and smaller levels of aspartate, glycine, and polar amino acids. These aragonitic layers also had lower ratios of aspartate to glutamate, serine to threonine, and glycine to alanine.

The amino acid compositions were very similar in the two species with shells of the same ultrastructure, in contrast to the differences between the homogeneous and composite-prismatic, and the complex and crossed-lamellar layers (Tables 3, 4). Although the compositions of the latter pair of layers were very similar, slight differences were detected between the former pair.

The homogeneous and composite-prismatic layers of both *Mercenaria stimpsoni* and *Meretrix lusoria* contained higher amounts of aspartate and hydroxy amino acids and lower amounts of proline and alanine, combined with higher

Table 4

Mean value and standard deviation of levels of each amino acid in the Ca-binding components in the six shell layers. Values are calculated for a total of five species in the case of both the nacreous and primsatic layers, three for the foliated, and two for the remaining three layers. —, not detected or not calculated.

	Nacreous		Prismatic		Foliated		Homogeneous		Composite-prismatic		Crossed-lamellar	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Asx	27.28	1.04	27.80	1.53	26.36	5.38	13.36	1.31	17.72	1.22	11.79	0.56
Thr	4.14	1.06	3.57	0.68	1.92	0.38	7.54	0.34	7.36	0.53	5.18	0.41
Ser	6.63	1.07	8.19	2.29	18.41	1.49	7.89	0.94	7.90	0.11	5.50	0.23
Glx	9.66	0.61	9.85	0.99	5.66	0.57	10.69	0.56	11.48	0.98	10.77	0.82
Pro	6.02	0.92	5.09	0.19	1.81	0.74	9.05	0.31	9.63	0.76	15.91	1.31
Gly	19.67	2.89	20.26	2.94	28.80	2.86	9.65	0.20	13.43	0.07	14.52	1.41
Ala	6.80	0.61	4.34	1.13	5.85	1.18	6.14	0.57	7.86	0.62	11.63	0.86
Cys	0.81	0.49	0.88	0.56	0.61	0.48	1.77	0.26	1.01	0.13	0.76	0.39
Val	3.40	0.64	2.61	0.28	1.15	0.33	5.18	0.56	4.22	0.32	4.94	0.32
Met	0.82	0.50	—[b]	—	—	—	0.83	0.26	0.13	0.08	0.48	0.24
Ile	1.85	0.46	1.58	0.42	0.32	0.11	3.54	0.10	2.44	0.56	3.10	0.41
Leu	3.56	0.78	2.95	0.46	2.04	0.56	3.95	0.10	4.22	0.10	4.64	0.45
Tyr	1.53	0.47	3.10	3.07	2.36	0.68	3.23	0.20	2.29	0.29	0.74	0.39
Phe	2.57	0.34	2.59	0.79	0.51	0.27	3.31	0.70	2.27	0.23	2.72	0.30
Lys	2.44	0.73	2.93	1.41	1.19	0.33	7.96	0.43	4.93	0.18	3.69	0.67
His	0.81	0.33	1.41	0.59	0.32	0.07	3.37	0.97	1.20	0.10	1.49	1.10
Arg	2.31	0.43	2.73	0.70	2.18	1.00	2.60	0.28	1.94	0.56	2.92	0.56

ratios of aspartate to glutamate, glycine to alanine, and aspartate to glycine than were found in the complex and crossed-lamellar layers. Between the homogeneous and composite-prismatic shell layers, differences were apparent in the amounts of aspartate, glycine and alanine, which were concentrated in the composite-prismatic layer, and in the proportions of aromatic and basic amino acids, which were concentrated in the homogeneous layer. The compositions of the Ca-binding components in the complex and crossed-lamellar layers of *Glycymeris yessoensis* and *Anadara broughtonii* were very similar to one another. Proline was the most abundant residue, accounting for nearly 14% or more of the total amino acid residues in each layer. Next in abundance was glycine, comprising about 13 to 16% of the total, followed by alanine, serine, tyrosine, and polar amino acids. Ratios of glycine to alanine, and of aspartate to glycine, were lower in the complex and the crossed-lamellar layers than in the Ca-binding components in the other layers.

SDS-Polyacrylamide Gel Electrophoresis and Isoelectric focusing

The Ca-binding components from each shell layer were subjected to SDS-Polyacrylamide gel electrophoresis and isoelectric focusing. No bands were observed after staining by Coomassie Brilliant Blue, regardless of sample concentration.

Infrared Spectroscopic Analysis

The infrared spectrum of the Ca-binding components in the nacreous layer of *Turbo cornutus* is shown in Figure 3. Peaks are seen at the position of 3280 cm^{-1}, 3070 cm^{-1}, 1658 cm^{-1}, 1534 cm^{-1}, 1230 cm^{-1}, 700 cm^{-1}, and 610 cm^{-1}. The absorptions at 3280 cm^{-1}, 3070 cm^{-1}, 1658 cm^{-1}, 700 cm^{-1}, and 610 cm^{-1} correspond to those of Amides A, B, I, and IV, respectively. However, one cannot determine whether the absorptions at 1534 cm^{-1} and 1230 cm^{-1} are those of Amide II and III or of carbohydrate. Additional peaks were seen at the positions of 1080 cm^{-1} and 890 cm^{-1} and might be due to absorbance by carbohydrates.

Quantitative Analysis of Phosphate and Hexose

Only trace amounts of phosphate were detected from all the samples examined. The amount of hexose was 2 to 3.5% (by weight) in the samples of the nacreous and foliated layers, and 0.5 to 1% of the remaining shell layers.

DISCUSSION

Ca-binding components were isolated from all the specimens examined. Although amino acid analysis showed that their main constituents were acidic amino acids, the exact isoelectric points of the Ca-binding components have not yet been determined because of the problem in staining gels after isoelectrofocusing. The data from the infrared spectroscopic analysis combined with the data from the quantitative analysis of hexose also demonstrated carbohydrates in the Ca-binding components. These results indicate that the Ca-binding components are composed primarily of highly acidic glycoproteins.

Gel exclusion chromatography indicated that the size of the Ca-binding glycoprotein was in the range of 50,000 to

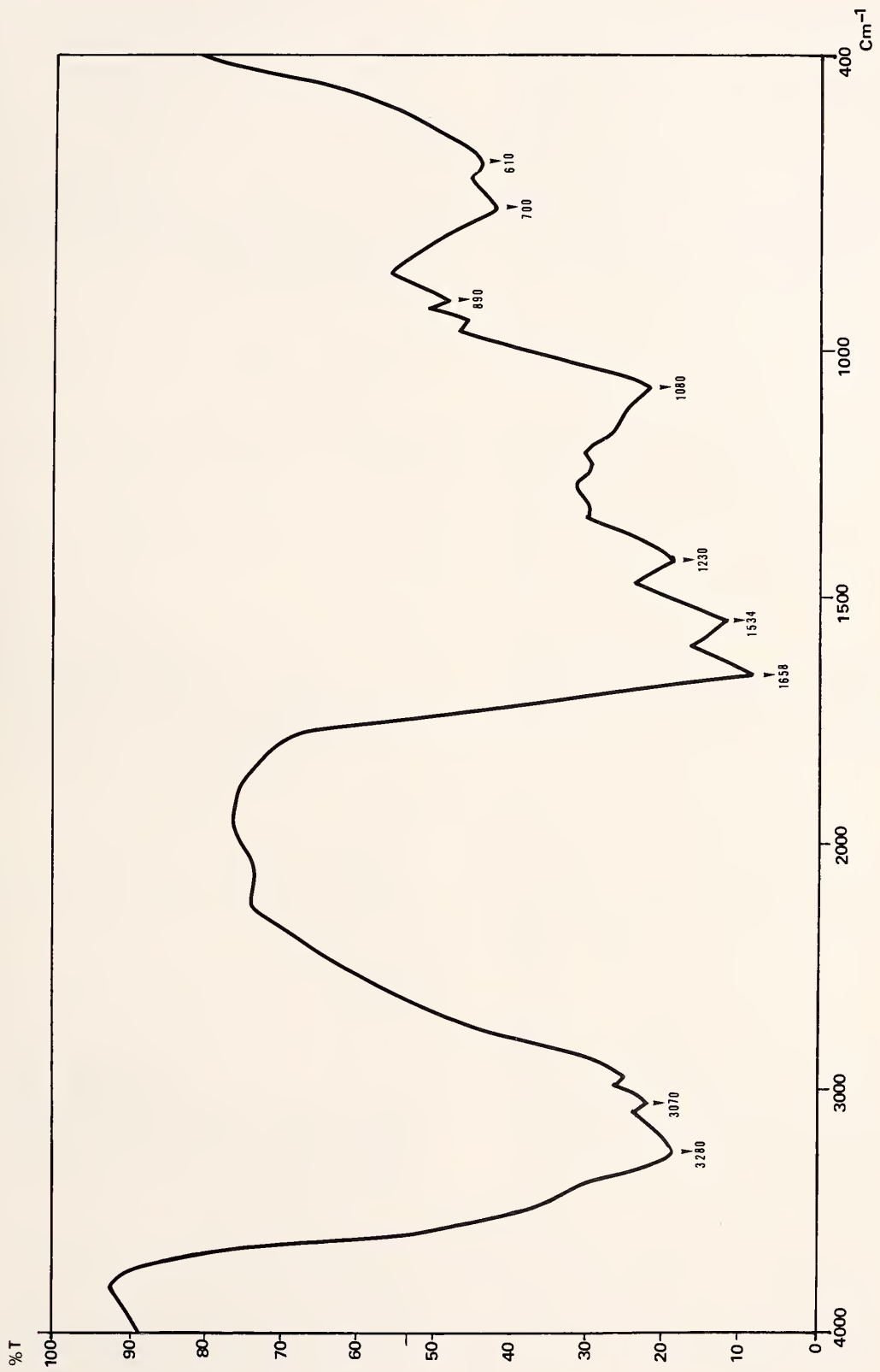

Figure 3

Infrared spectrum of the Ca-binding glycoproteins in the nacreous layer of *Turbo cornutus*.

70,000 daltons. Although WEINER *et al.* (1977), using SDS-polyacrylamide gel electrophoresis, showed the presence of components with discrete molecular weights in the SM of several molluscan species, no visible bands could be detected by Coomassie Blue staining in the present study. The lack of detection may be due to the low proportion of basic amino acids in the glycoprotein or to some co-existing carbohydrate moiety; more suitable conditions for electrophoresis and staining must be found in the future. Although the purity of the glycoproteins could not be checked electrophoretically, further fractionation of the Ca-binding components was impossible, at least by column chromatography. The highly acidic nature of the components was also clearly shown by chromatography on DEAE Sephacel: components could be released from the anion exchange resin only at the high ionic strength of 0.4 to 0.6 *M* NaCl. In previous studies, heterogeneous components were identified in the SM by ion exchange chromatography, by adjusting the concentrations of NaCl (usually less than 0.3 *M*) at which the proteins eluted (WEINER, 1979; WEINER *et al.*, 1977). The detection of components at lower ionic strength than in the present study could result from the binding of EDTA to the protein. This possibility cannot be ruled out, since a large quantity of EDTA may still remain after gel chromatography (SAMATA & MATSUDA, 1986).[1]

In spite of the high degree of similarity in the behavior of the Ca-binding components during column chromatography, the amino acid composition of the components varied primarily according to the ultrastructures of the shells. The samples used for this analysis included 12 species of three orders of Mollusca, which were composed of eight types of shell ultrastructure. The compositions of the Ca-binding glycoproteins in both the nacreous and prismatic layers of five species of Gastropoda and Bivalvia were very similar, despite their widely separated taxonomic positions. On the contrary, the Ca-binding glycoproteins of nine species of Bivalvia, whose shells are composed of eight types of ultrastructure, had unique compositions according to the ultrastructure. Moreover, the compositions of the Ca-binding glycoproteins in the homogeneous and composite-prismatic layers differed from each other. These data imply that the composition of the Ca-binding glycoproteins may vary primarily according to shell ultrastructure, and not to taxonomic position.

Based on the amino acid composition, the Ca-binding glycoproteins can be classified into three types: (1) those of the nacreous and prismatic layers; (2) those of the foliated and chalky layers; and (3) those of the remaining four shell layers. These three types differ primarily with respect to the amounts of aspartate, serine, proline, glycine, and alanine (Figure 4). The amounts of threonine, glutamate, and lysine also vary but to a lesser extent. Almost all other amino acids, whose levels were relatively low, showed slight variations according to shell ultrastructure. In the third type of ultrastructure, two additional subtypes can be distinguished between the homogeneous and composite-prismatic layers, and the complex and crossed-lamellar layers. The difference was most remarkable in terms of the amounts of proline, tyrosine, and hydroxy amino acids. Moreover, the compositions of the Ca-binding glycoproteins differ slightly, in terms of the amounts of aspartate, glycine, alanine, and basic amino acids, between the homogeneous and composite-prismatic layer (Figure 4).

Amino acid analyses by previous workers have been carried out mainly on the unfractionated ISM regardless of ultrastructure. Therefore, the previous results are not comparable to those obtained for the Ca-binding glycoproteins in the present study. For example, MEENAKSHI *et al.* (1971) found the ISM in the nacreous layer to be rich in glycine and alanine, in contrast to the high levels of aspartate of the Ca-binding glycoprotein in the same layer.

The organic matrix has been suggested to be responsible for initiating, regulating, and limiting mineral growth (WEINER & HOOD, 1975; KRAMPITZ *et al.*, 1976; WHEELER *et al.*, 1988). For understanding the function of the matrix, interactions between the SM and the ISM must be elucidated. SAMATA (1988a) indicated the distinctive difference in composition between the SM and the ISM of both the nacreous and prismatic layers. Moreover, the composition of the ISM was also far separated between the two layers. On the other hand, the compositions of the two matrices were very close in other shell layers (SAMATA, 1988b). Thus, the organic matrix may participate in calcification in different ways between these two groups of shell ultrastructures, *i.e.*, the nacreous and prismatic layers and the remaining six layers. In the nacreous and prismatic layers, the ISM may limit crystal growth, and thus regulate the orientation of crystals to the arrangement of the nacreous and prismatic layers, because the amino acid compositions of the Ca-binding glycoproteins in the SM did not differ significantly. Whether the Ca-binding glycoproteins in these layers play a positive role, such as transporting Ca-ions or initiating crystal nuclei, or serve as a

[1] In the present study, HCl was used for decalcification of molluscan shells in order to avoid contamination that may occur by the use of EDTA. Although dilute acid could introduce cleavage in peptide bonds and denaturation in protein structure, KASAI & OHTA (1981) reported that the amino acid composition of the matrix varied little when shells were decalcified by EDTA or dilute acids. In addition, high capacity of Ca-binding could still be detected in matrix that was decalcified by HCl (SAMATA, 1988a). These results imply that organic matrix in molluscan shells is fairly resistant to acids. Furthermore, the use of EDTA has certain disadvantages. EDTA binds tightly to the inner wall of dialysis tubing, and also forms unidentifiable biopolymers with proteins, which are difficult to separate (SAMATA & MATSUDA, 1986). All the common amino acids are present in EDTA of special grade (Wako Pure Chemicals, Osaka, Japan) in amounts that cannot be ignored and EDTA ia also capable of binding Ca^{2+}-ions (SAMATA & MATSUDA, 1986).

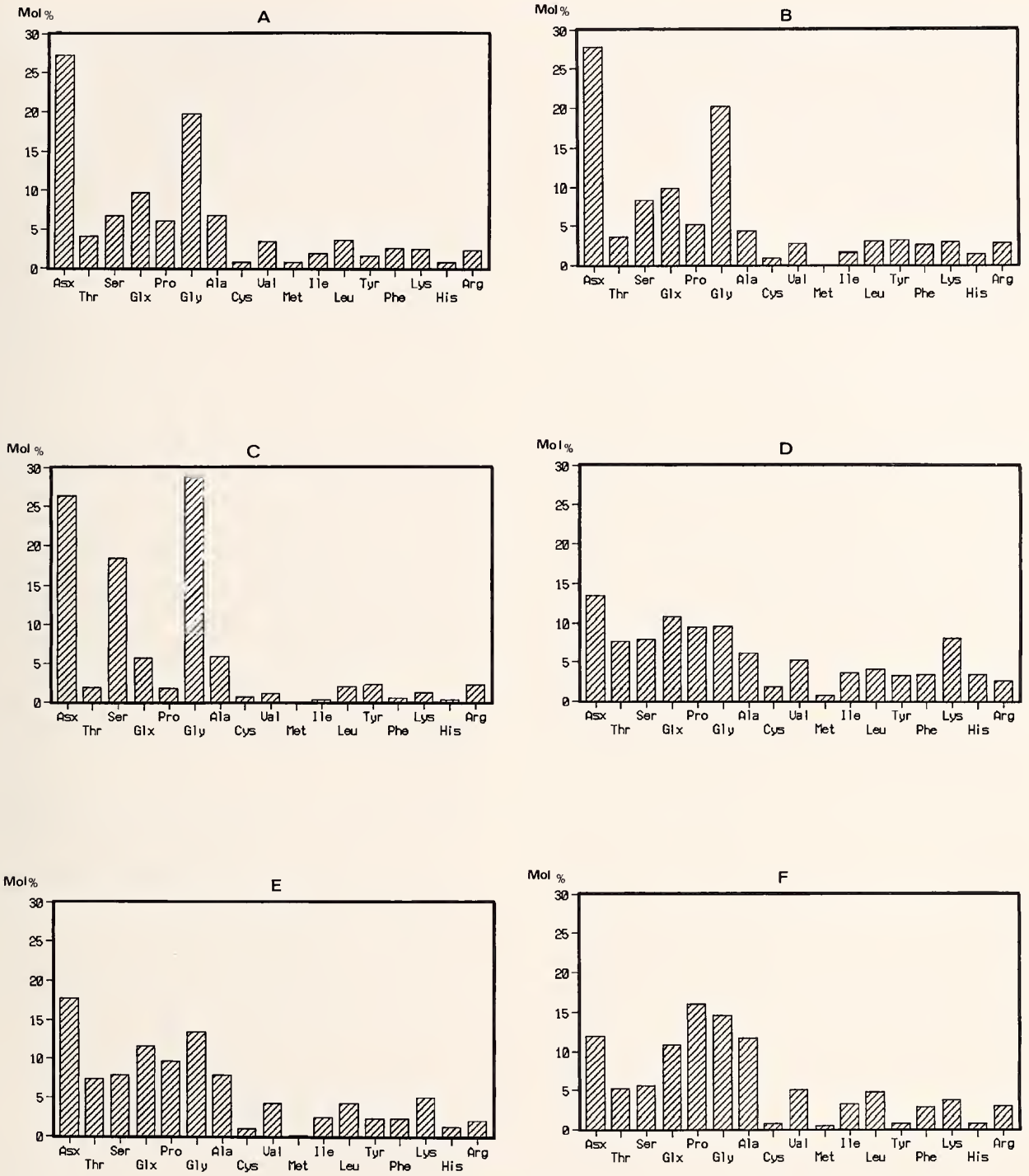

Figure 4

Diagrams of the amino acid compositions of the Ca-binding glycoproteins in six different shell layers. A. Amino acid compositions of the Ca-binding glycoproteins in the nacreous layer of *Pinctada martensii*. B. Amino acid compositions of the Ca-binding glycoproteins in the prismatic layer of *Pinctada martensii*. C. Amino acid compositions of the Ca-binding glycoproteins in the foliated layer of *Crassostrea gigas*. D. Amino acid compositions of the Ca-binding glycoproteins in the homogeneous layer of *Meretrix lusoria*. E. Amino acid compositions of the Ca-binding glycoproteins in composite-prismatic layer of *Meretrix lusoria*. F. Amino acid compositions of the Ca-binding glycoproteins in the complex and crossed-lamellar layers of *Glycymeris yessoensis*.